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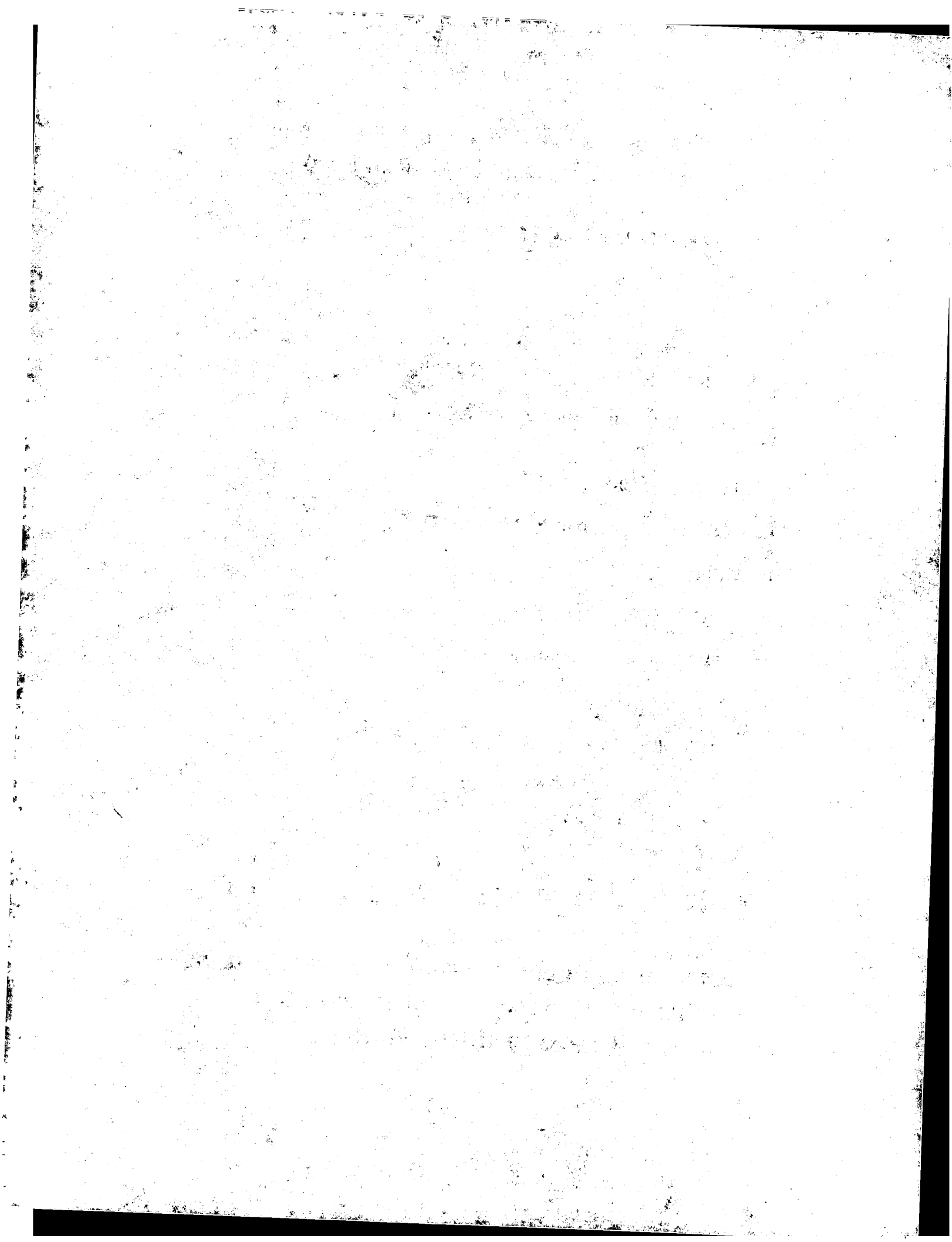
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(54) **Chromatographic adsorbent particles, methods of their preparation, and their use as a solid phase matrix in a fluid bed reactor.**

(57) Chromatographic adsorbent particles (10) having covalently bound at least one active substance for binding molecules in a liquid chromatographic fluid bed process; said adsorbent particles being constituted by a porous composite material having pores allowing access to the interior of the composite material of said molecules; wherein the porous composite material consists of a conglomerate having controlled relative density; said conglomerate consisting of:

- (i) at least two density controlling basic particles (11) of amorphous silica, quartz, or glass; and
- (ii) a matrix formed by consolidating at least one conglomerating agent (12) selected from the group consisting of natural and synthetic polysaccharides and other carbohydrate based polymers with the proviso that the conglomerating agent is not β -1,3-glucan.

The at least two density controlling basic particles are dispersed in said matrix; the size range of the adsorbent particles is controlled; said density and said size range are selected to provide desired floatation/sedimentation properties of said adsorbent particles in the liquid in said fluid bed process; and the at least one active substance is covalently bound to said matrix.

Methods of preparing such adsorbent particles, their use as a solid phase matrix in a fluid bed reactor, and particularly their use for distributing the liquid in the fluid bed of such a reactor.

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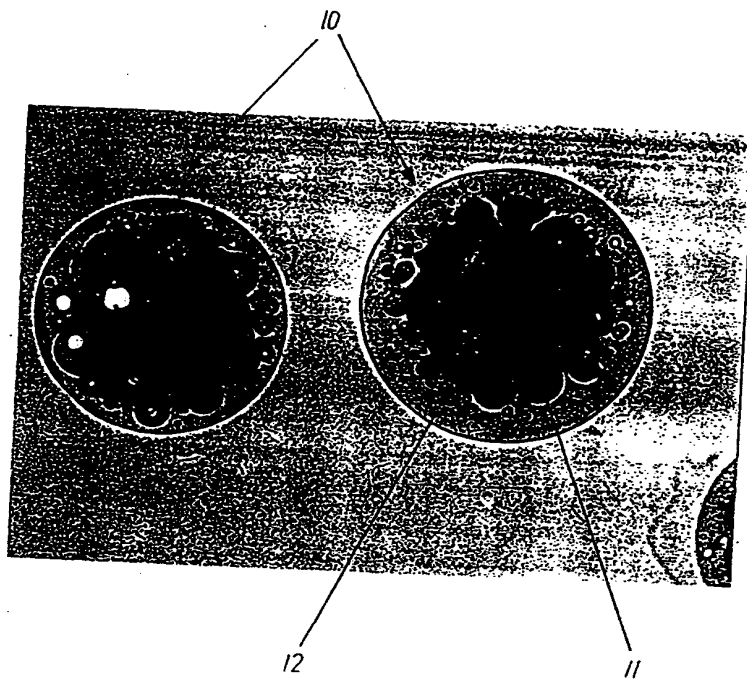


FIG. 1B

1. BACKGROUND OF THE INVENTION*The Technical Field*

5 The present invention relates to chromatographic adsorbent particles according to the pre-characterizing part of claim 1; methods of their preparation; and their use as a solid phase matrix in a fluid bed reactor.

"Definition of Expressions"

10 In the present context the expression "conglomerate" is intended to designate a composite of basic particles, which may comprise particles of different types and sizes, held together by conglomerating agents. Conglomerates may be of various sizes, and shapes and should preferably exhibit various degrees of mechanical rigidity depending on the application. Further, conglomerates may be chemically active or may be chemically inactive under the conditions applied.

15 The expression "conglomerate of controlled relative density" is intended to designate a conglomerate or a conglomerate particle for which in particular the basic particles are chosen in predetermined amounts to provide a certain relative density of the conglomerate with respect to the liquid in which an active substance or another constituent of the conglomerate is to be used so that the floatability or sedimentation, respectively, is controlled.

20 Thus, chromatographic adsorbent particles according to the invention are intentionally designed with respect to the density of the medium for their particular purpose of application, including proper consideration of the influence of their sizes on their floating or sedimentation properties. In other media, e.g. during preparation or during storage under e.g. dry conditions, the chromatographic adsorbent particles may have a density different from that in the liquid medium of use.

25 In the present context the expression "active substance" should be taken in a very broad sense comprising agents having desired properties for their particular purpose of application, e.g. adsorbents, ligands, reagents, and natural substances covalently bound to the conglomerate of controlled density.

"Industrial Applicability"

30 Chromatographic adsorbent particles having covalently bound at least one active substance are used in a wide variety of applications in chromatographic processes, e.g. high performance liquid chromatography, gel filtration, ion exchange and affinity chromatography, e.g. carrying adsorbents; diagnostic processes, e.g. carrying adsorbents for blood purification, dye chromatographic processes for albumine purification; and
35 prophylactic processes, e.g. carrying immobilized antibodies or antigens in extracorporeal circulations for removal of antigens or antibodies, bacterial toxins or other toxins, and autoimmune diseases.

Prior Art Disclosure

40 There are numerous disclosures in the prior art concerning particles prepared from organic and inorganic materials.

However, chromatographic adsorbent particles as claimed in claim 1 have apparently never been disclosed.

"Coated Particles"

45 Kuraray Co., Ltd., C.A. 98:157436t discloses beads, particles, fibres, sheets, and tubes of glass, activated carbon, silica, alumina or high molecular weight substances coated with copolymers of acrylates and carboxylic acids or amines to form selective adsorbent carriers or supports for use in selective
50 electrodes or in column chromatography.

Sakuma et al., C.A. 111:74363c, disclose glass or polymer spheres coated with hydroxyapatite for use as a stationary phase for column chromatography.

55 EP-A-0266580 discloses a method for coating solid particles with a hydrophilic gel preferably agarose for various separating processes in packed columns based on adsorbent groups, e.g. ion exchanging groups, hydrophobic groups, or groups with biospecificity chemically bound to a gel. Such coating may be provided by mixing hydrophilic solid particles with a gel-forming substance above the gelling temperature in which each individual particle is coated, separated from each other, and cooled below the gelling temperature, essentially to stabilize the particles against the high pressure in e.g. HPLC applications.

Generally, all of the above mentioned coated particles are provided by coating individual particles made of the same material and having the same density.

"Hollow Particles"

5

US 4698317 discloses hollow microspherical glass particles having open pores, and being prepared by spray thermal decomposition of a solution, in an aqueous organic solvent, wherein the water content promotes open pore formation.

US 2797201 discloses substantially spherical, hollow particles having a "thin, strong skin" being prepared by thermal treatment of droplets of a solution of a film forming material, e.g. an organic polymer such as a phenol-formaldehyde resin, and optionally further containing a "blowing agent", i.e. an agent generating gas at the elevated temperature of the thermal treatment.

GB 2151601B discloses porous hollow particles of an inorganic material and a composite material comprising such particles supporting a selected substance such as a chromatographic organic gel. The porous hollow particles may be formed by coating a fugitive core material, e.g. organic resin beads or alginate spheres, with inorganic material, and then heating to remove the fugitive core material. Further, GB 2151602B discloses closely similar particles wherein a magnetic material, such as ferric oxide, nickel oxide or cobalt oxide, is incorporated in the inorganic shell of the particle.

The 3M Corporation (USA) markets a number of types of substantially impermeable, hollow microspheres of silicious material. For example synthetically manufactured soda-lime borosilicate glass microspheres marketed by 3M in a variety of size fractions. Also, permeable hollow spheres of siliceous material derived from fly-ash are provided by Fillite Ltd., Runcorn, England. However, none of the commercially available microspheres are conglomerates of controlled relative density within the meaning of the present invention.

EP-A-0021563 discloses a material suitable for thermosetting which includes a collection of hollow particles adhesively mixed with a thermosetting resin and which material may be converted by thermosetting in to a fused solid mass having a density not greater than 0.5 g/cm³.

"Pharmaceutical Dosage Forms"

30

GB-A-2196252 discloses an oral, solid, pharmaceutical dosage form comprising conventional matrix binders including starch and cellulose, or their derivatives, and a pharmaceutically acceptable weighting agent, including inorganic compounds such as salts, oxides, or hydroxides of a metal, e.g. barium sulphate or ferrous oxide, suitable for oral administration to humans and for controlled release of a pharmaceutically active ingredient into the stomach. The controlled release unit may have any chosen density from about 2 g/ml to about 6 g/ml and may in case of a conventional pellet have a size from about 1 to about 1.4 mm, and in case of a tablet a size above 10 mm. Nothing is disclosed nor suggested about non-solid i.e. permeable or porous conglomerates of controlled relative density within the meaning of the present invention. Furthermore, the described pharmaceutical dosage form consists of solid particles comprising a binder and a weighting agent soluble in gastric fluid which makes the pellet or tablet disintegrate shortly after ingestion.

"Fluid Bed Particles"

Generally, for a large number of applications, the active substance for binding molecules in a liquid chromatographic fluid bed process may only temporarily be available or accessible at the right places in the fluid. Thus, for chromatographic adsorbent particles carrying active substances and often showing large variations in dispersion properties, e.g. sedimentation or floatation, the active substances may be carried in an uncontrolled manner e.g. down- or upwards in relation to the liquid depending on the relative density of the adsorbent particle.

In fluid bed reactors partially solving the problems of packed bed columns, i.e. the problems of suspended matter clogging up the solid-phase bed which increases the back pressures and compresses the bed disturbing the flow through the bed, the adsorbent particles are carrying the active substance in a free, fluid phase by applying a flow having an opposite direction to the direction of the relative movement of the adsorbent particle. Thus, adsorbent particles having a density larger than the fluid and moving downwards due to gravity may be kept in a free, fluid phase by an upwards flow of fluid. Also, adsorbent particles having a density less than the fluid and thus moving upwards may due to buoyancy be kept in a free, fluid phase by a downwards flow of fluid.

For fluid bed liquid chromatographic processes, the density of the solid-phase adsorbent particle is very important in controlling bed properties. However, up to now, the design of solid-phase adsorbent particles has been limited by the available material.

Generally, particles may either be designed to be impermeable to the fluid, in which case the available surface area per unit volume is small; or particles may be designed to be permeable to the fluid, in which case the material chosen has to have the correct density *per se*. Unfortunately, the most interesting materials for many applications, e.g. materials such as natural and synthetic polysaccharides like agar, alginates, carrageenans, agarose, dextran, modified starches, and celluloses for chromatographic purification of proteins in packed bed columns are not of suitable density *per se*. Therefore, these materials are difficult to apply in fluid bed reactors.

Certain types of organic polymers and certain types of silica based materials may be produced to provide adsorbent particles of suitable density, but such adsorbent particles may not at the same time be suitable active substances, e.g. for protein purification procedures, where such materials may provide low permeability, non-specific interactions and denature bound proteins. Further, for such polymers, it may be difficult and expensive to design derivatisation schemes for affinity chromatography media. Also, certain types of permeable silica particles have been used for fluid bed applications. However, the properties of these materials are far from optimal. Thus, the materials are instable at pH above 7, fragile to shear forces, and provide non-specific interactions.

20 "Particles Having Active Substances Restricted to the Surface"

US 4032407 discloses a tapered bed bioreactor applying immobilized biological catalysts or enzymatic systems on fluidizable particulate support materials consisting of coal, alumina, sand, and glass, i.e. materials heavier than the fluid, particularly an aqueous fluid.

25 EP-A-0175568 discloses a three phase fluidized bed bioreactor process comprising purifying effluents in a three phase fluidized bed comprising solid particles being made by mixing a binder with an inorganic material based on aluminum silicate, granulating the resulting mixture, and firing the granules to sinter them. The specific gravity of the sintered granules is adjusted to fall into a specific range from 1.2 to 2.0 by varying the mixing ratio of inorganic powdery materials based on aluminum and binders, said sintered granules having a diameter from 0.1 to 5 mm.

EP-A-0025309 discloses a downflow fluid bed bioreactor applying biota attached to carrier particles consisting of cork, wood, plastic particles, hollow glass beads or other light weight material and having a specific gravity which is less than that of a liquid sprayed onto the upper part of a fluid bed of suspended carrier particles and conducted downward through the bed.

35 These three disclosures describe particulate support materials to which the attachment of the active substance is restricted to the surface of the particles limiting the amount of active substance to be obtained per unit volume compared to chromatographic adsorbent particles allowing the active substance to be attached within the particle. Thus, in many applications, it is important to have specifically designed chromatographic adsorbent particles which are able to carry as large an amount of active substance per unit volume as possible, however, such chromatographic adsorbent particles are not available in the prior art.

Thus, in great many applications of active substances in liquid chromatographic fluid bed processes, there is a need for materials of controlled relative density carrying the active substances.

45 "Distribution of Liquid in a Fluid Bed"

A disadvantage of distributing an introduced liquid in a fluid bed reactor by spraying is the formation of channels in the bed by fluid rays.

EP-A-0005650 discloses an up-flow fluid bed reactor having fluidizing fluid flow distributors at the bottom thereof providing flow paths to avoid turbulence effects. Besides requiring complicated flow paths, a disadvantage of such a distributor is that it may be clogged by particulate matter.

"Removal of Oil on Water Surfaces"

55 US 4142969 discloses an oleospecific hydrophobic composition comprising an intimate mixture of expanded volcanic glass consisting of perlite, a cellulose fiber, and a water repellent sizing consisting of asphalt; and a method of sorbing oleaginous compounds e.g. in selectively removing oil from the surface of water. The constituents are incorporated into a homogeneous product by a wet process, dried in an oven

until essentially all moisture has been removed, and then ground up into a fluffy low density material. Nothing is disclosed nor suggested about chromatographic adsorbent particles as claimed in claim 1.

2. DISCLOSURE OF THE INVENTION

(a) Chromatographic Adsorbent Particles

It is the object of the present invention to provide improved chromatographic adsorbent particles as defined in the pre-characterizing part of claim 1. Particularly, it is the object of the present invention to provide chromatographic adsorbent particles avoiding the disadvantages of known chromatographic adsorbent particles e.g. problems of uncontrolled sedimentation or floatation of the chromatographic adsorbent particles, the poor selectivity and capacity of chromatographic adsorbent particles, and to provide the possibility of simultaneously designing and controlling the properties of the active substance and chromatographic adsorbent particles.

According to the invention this object is fulfilled by providing chromatographic adsorbent particles as defined in the pre-characterizing part of claim 1 which adsorbent particles are characterized by the features of the characterizing part of claim 1.

According to the invention, chromatographic adsorbent particles may further comprise other substances such as additives, fillings, softeners, etc., and possibly comprising a suitable surface coating.

In its broadest aspect the density may be controlled by selecting at least two density controlling basic particles of amorphous silica, quartz, or glass from a group of particles consisting of particles of very low density, particularly hollow and impermeable particles having shells of suitable material and properties; however, non-hollow particles may be chosen when appropriate; and particles of very high density, e.g. particles based on suitable heavy elements or compounds.

Generally, the invention provides a new type of chromatographic adsorbent particles comprising conglomerates of controlled relative density, selectivity, and capacity in terms of controllable interior surface areas and materials e.g. materials having specific chemical and/or mechanical properties. Thus, compared with known chromatographic adsorbent particles for fluid and packed bed reactors, chromatographic adsorbent particles according to the invention can surprisingly be designed to have a number of advantages not previously obtained.

According to the invention, chromatographic adsorbent particles can be designed to have a controlled relative density independent of the active substances and the conglomerating agents; heavy particles can be made light, and vice versa, within a wide range of particle sizes; the density can be controlled within very broad limits, e.g. the density of a known material can be controlled for a specific application; the volume percentage of the conglomerating agent can be controlled according to the application; the total size of the final chromatographic adsorbent particles can be controlled contrary to known particles having uncontrollable sizes for specific densities suitable for particular rising and falling velocities. Further, chromatographic adsorbent particles according to the invention have a relative larger capacity, i.e. a larger accessible volume, compared to e.g. known impermeable chromatographic adsorbent particles. Also, in preparing such known impermeable chromatographic adsorbent particles, the active substances to be applied are limited, e.g. limited to substances that can be attached to the particle surface. Further, contrary to known chromatographic adsorbent particles having a given mechanical strength and density, the elasticity and the mechanical strength of chromatographic adsorbent particles according to the invention can be controlled independently of the density. Also, pore sizes and e.g. biocompatibility can be controlled independently of the density in order to allow access to the interior of the chromatographic adsorbent particles and to avoid denaturation e.g. of proteins.

Preferred embodiments are defined in the claims 2-18.

(b) Methods of Preparing Chromatographic Adsorbent Particles

Preparation of chromatographic adsorbent particles according to the invention may be obtained by various methods known *per se*, e.g. block polymerisation of monomers; suspension polymerisation of monomers; block or suspension gelation of gelforming materials, e.g. by heating and cooling (e.g. of agarose) or by addition of gelation "catalysts" (e.g. adding a suitable metal ion to alginates or carrageenans); block or suspension cross-linking of suitable soluble materials (e.g. cross-linking of dextrans, celluloses, or starches with e.g. epichlorohydrine or divinyl sulfone); mixed procedures e.g. polymerisation and gelation; spraying procedures; and fluid bed coating of density controlling basic particles.

For particularly preferred embodiments according to the invention, chromatographic adsorbent particles may be obtained by cooling emulsions of density controlling basic particles suspended in conglomerating agents in heated oil solvents; or by suspending density controlling basic particles and active substance in a suitable monomer or copolymer solution followed by polymerisation.

"Preparation by Gelation/Polymerisation in the Emulsified State"

A method of preparing chromatographic adsorbent particles according to the invention is defined in claim 19.

Preferred embodiments are defined in claims 20 and 21.

"Preparation by Gelation/Polymerisation in the Block State"

Another method of preparing chromatographic adsorbent particles according to the invention is defined in claim 22.

Preferred embodiments are defined in claims 23 and 24.

For polysaccharides such as agarose and agar, i.e. materials melting at high temperatures and solidifying at low temperatures, the conglomerating means is by heating/cooling.

Particularly for heavily charged polysaccharides and polymers such as alginates and carrageenans, the conglomerating means is non-covalent cross-bonding by addition of a suitable metal ion. However, for polysaccharides in general, e.g. cellulose and its derivatives, and polymers containing e.g. amino, hydroxyl, thiol, and carboxy groups, the conglomerating means is covalent cross-bonding by addition of a suitable cross-bonding agent, e.g. epichlorohydrine, divinyl sulfon, bisepoxyranes, dibromopropanol, glutaric dialdehyde, diamines, and other bifunctional agents.

Also, the above mentioned conglomerating means may be combined in specific cases such as the preparation of conglomerates of agarose-acryl-derivatives and cross-bonded mixtures of agarose and dextran.

Further, in the above mentioned block polymerisation, the segregation step of the polymer block may be obtained by methods known *per se*, e.g. by granulation and sieving.

(c) The Use of Chromatographic Adsorbent Particles

"Solid Phase Matrix in Fluid Bed Reactors"

The invention also relates to the use of chromatographic adsorbent particles according to the invention as a solid phase matrix in fluid bed reactors.

Generally, a fluid bed reactor may comprise a vertical reactor with an inlet, an outlet, a fluid bed of particles, and a liquid. The liquid is introduced at the inlet and dispersed, optionally through a gas head in case of down-flow reactors, on the bed of particles which are suspended and fluidized by the liquid. The liquid is conducted through the bed and a pool of reacted and/or unreacted liquid is let out at the outlet.

Down-flow fluid bed reactors have liquid inlet at the top of the reactor and fluid bed particles of specific gravity less than that of the liquid.

Up-flow fluid reactors have liquid inlet at the bottom of the reactor and fluid bed particles of specific gravity larger than that of the liquid.

The suspended particles may be reactive or may carry immobilized reactive components selected for solid phase chemical or physical processes with one or more components of the fluid in procedures such as enzymatic reactions; fermentation; ion-exchange and affinity chromatography; filtration; adsorption; catalysis; immunosorption; solidphase peptide and protein synthesis; and microbiological growth of microorganisms.

It is an object of the invention to provide the use of chromatographic adsorbent particles according to the invention in solid-phase chemical processes in continuous fluid bed reactors particularly for separation of proteins.

The use of chromatographic adsorbent particles according to the invention, including β -1,3-glucan as a conglomerating agent, as a solid phase matrix in a fluid bed reactor is defined in claim 25.

Preferred uses of the chromatographic adsorbent particles are defined in procedures as defined in claim 26.

Further, specifically preferred uses of the chromatographic adsorbent particles in a liquid down-flow fluid bed reactor and in a liquid up-flow fluid bed reactor are defined in claims 27 and 29, respectively.

"Distribution of Liquid in the Fluid Bed of a Liquid Fluid Bed Reactor"

Generally, in order to carry out solid phase chemical or physical processes in a fluid bed reactor, an even and smooth distribution of liquid in the fluid bed is desired. However, fluid bed reactors known in the art do not have means known *per se* to avoid formation of channels as well as unwanted turbulence in the particle bed.

It is another object of the present invention to provide the use of chromatographic adsorbent particles according to the invention in a fluid bed reactor for distributing a liquid in the fluid bed such that the liquid is distributed evenly and with minimal or no turbulence in the fluid bed.

The use of chromatographic adsorbent particles according to the invention or this particular purpose in a liquid down-flow fluid bed reactor and in a liquid up-flow fluid bed reactor are defined in claims 28 and 30, respectively.

According to the invention this is obtained e.g. by providing a liquid down-flow fluid bed reactor comprising a vertical reactor with an inlet, an outlet, and a fluid bed of particles, wherein the particles proximal to the liquid inlet are agitated, preferably by stirring with a mechanical stirrer which does not form a vortex, forming a turbulent zone of vigorously moving particles adjoining a non-turbulent zone where the particles are in stationary fluidized state, said turbulent zone having an extent determined by the degree of agitation the value of which is selected for a given flow of liquid, viscosity, and buoyancy of the particles, within a range from a degree of agitation where the particles move but do not mix to a degree of agitation where the particles mix throughout the fluid bed.

Also, the invention provides a similar method for an up-flow fluid bed reactor in which the extent of the turbulent zone is determined based on the sedimentation of the fluid bed particles instead of the buoyancy of the particles as in case of the down-flow fluid bed reactor.

It is known that fluids and solids can be mixed by agitation. However, to the applicants' knowledge, it is new to apply agitation to fluid bed reactors for the purpose of distributing the liquid flow in the fluid bed, particularly for a down-flow reactor.

Apparently, in the art, it has been anticipated that agitation of a fluid bed generates turbulence which may result in unwanted mixing of products and reactants, and in unwanted wear of the bed particles. However, according to the invention, these advantages can be limited considerably by agitating a part of the fluid bed, particularly the part of the bed proximal to the inlet of the liquid.

Particularly, for a down-flow fluidized fluid bed reactor it has turned out that agitation of the upper part of the fluid bed divides the bed in two zones:

- i) a turbulent zone with turbulence and vigorously moving particles; and
- ii) a non-turbulent zone with no turbulence and particles in a stationary fluidized state.

The two zones adjoin with a sharp interface across which the liquid flow is evenly distributed. The position of the sharp interface is controlled by the degree of agitation which is selected for a given flow of liquid, viscosity, and buoyancy /sedimentation of the particles. Thus, according to the invention, a turbulent zone provides an even distribution of liquid flow to the non-turbulent zone with minimal or no turbulence. A number of advantages are obtained.

For chromatographic applications, the dispersion of eluant is reduced, i.e. the width of the elution band is reduced. Further, formation of channels in the fluid bed is minimized.

According to the invention, agitation can be effected by stirring the fluid bed or by any agitation means, including mechanical agitation or gas injection.

According to the invention, the fluid bed particles can be different or of the same type for both the turbulent zone and the non-turbulent zone.

In a preferred embodiment, the turbulent zone may comprise inert particles of slightly different specific gravity than the particles of the non-turbulent zone. In this case, the inert particles positioned in the turbulent zone solely participate in the distribution of liquid, and not in the solid phase processes.

Generally, compared to packed bed techniques, fluid bed techniques, e.g. to be used in fluid bed chromatography, are better suitable to large scale primary purification of proteins as the steps of centrifugation and filtration can be avoided. Thus, the fluid bed techniques can be used immediately following the production of the protein, e.g. directly applying the produced extract or fermentation liquid to fluid bed purification and conversion. Accordingly, using chromatographic adsorbent particles according to the invention in fluid bed techniques, several advantages such as the control of the density, and the choice of materials to design the chemical and/or mechanical properties of the adsorbent particles, e.g. including cheaper basic materials, are obtained.

3. BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by reference to the examples given below and to Figures 1-6, wherein

- 5 Fig. 1A shows a 40X amplified photograph of conglomerates of agarose and glass spheres prepared according to Example 1(a);
- Fig. 1B shows a 40X amplified photograph of selected spherical conglomerates of agarose and glass spheres prepared according to Example 1(a);
- Fig. 2 illustrates a preferred embodiment of a fluid bed reactor;
- 10 Fig. 3 illustrates another preferred embodiment of a fluid bed reactor;
- Fig. 4A and 4B show perspective sketches of another preferred embodiment of a down flow fluid bed reactor;
- Fig. 5 illustrate the fluid bed particles of conglomerates according to the invention in a down flow fluid bed reactor; and
- 15 Fig. 6A-6D illustrate cross-sections along the lines VIB, VIC, VID, VIE in Fig. 5.

4. DETAILED DESCRIPTION

(a) *Controlled Relative Density of Chromatographic Adsorbent Particles*

20 Within the present context the expression "relative density chromatographic adsorbent" designates the density of the individual chromatographic adsorbent particles in the wet state, i.e. a state where the conglomerating agent is fully hydrated, but without any interstitial liquid between individual conglomerate particles. This means that the liquid in which the chromatographic adsorbent particles are used are
 25 determinant for the density of the chromatographic adsorbent particles in as much as this liquid penetrates into the volume of the conglomerating agent, solvates this and fill out the pores.

This relative density is determinant for the tendency of the particles to float or to sediment in a given liquid. The relative density of chromatographic adsorbent particles according to the invention is thus dependent on the solvated density of the conglomerating agent, the concentration of conglomerating agent,
 30 the density of the density controlling basic particles (impermeable to the liquid and substantially non-solvated) used to regulate the density and the concentration of these.

The density of the solvated phase, i.e. the volume occupied by the conglomerating agent and the active substance will usually be dependent on the specific application of the particles and thus not allowable to be regulated by variation of the concentration of conglomerating agent. Therefore, according to the invention
 35 the density of the chromatographic adsorbent particles is regulated by the addition of density controlling basic particles having a density free of choice with respect to the functionality of the chromatographic adsorbent particles and also having a final concentration in the chromatographic adsorbent particles free of choice with respect to the functionality, i.e. the functionality of the active principle within the volume of the conglomerating agent is not disturbed by the density and concentration of the density controlling basic
 40 particles.

A crude estimate of the final density as a function of the concentration of density controlling basic particles can be found by the following equation:

$$\text{Density of chromatographic adsorbent particles} = ((d_c \times v_c) + (d_b \times v_b)) / (v_c + v_b)$$

45

d_c = density of solvated conglomerating phase

d_b = density of density controlling basic particles

v_c = volume occupied by solvated conglomerating phase

50 v_b = volume occupied by density controlling basic particles.

Differences in the degree of solvation occurring in different solvents have to be corrected for. Thus, for certain conglomerating agents, e.g. heavily charged polymers for ionexchange chromatography, the degree of solvation, i.e. the volume of liquid taken up per gram dry weight, may differ with several hundred percent in fluids with different ionic strength or pH.

55 By way of example the density of chromatographic adsorbent particles comprising agarose as the conglomerating agent and hollow glass spheres as density controlling basic particles is regulated by the addition of hollow glass spheres to the liquified agarose, the amount added (for example measured as gram hollow glass spheres per ml agarose) being determinant for the density of the final chromatographic

adsorbent particles.

Assuming a density of the agarose phase to be 1.0 g/ml and the volume used to be one liter (1000 ml) and the density of the hollow glass spheres to be 0.2 g/ml and the amount used to be 100 g (corresponding to 500 ml) the calculated density would be:

$$((1.0 \times 1000) + (0.2 \times 500)) / (1000 + 500) = 0.73 \text{ g/ml}$$

If only 50 g of hollow glass beads were added the calculated density would be:

$$((1.0 \times 1000) + (0.2 \times 250)) / (1000 + 250) = 0.84 \text{ g/ml}$$

If instead of the hollow glass spheres, the basic particles used were solid glass spheres with a density of 2.5 g/ml and 500 g were used to the same amount of agarose, the calculated density would be:

$$((1.0 \times 1000) + (2.5 \times 200)) / (1000 + 200) = 1.25 \text{ g/ml}$$

"Concentration of Density Controlling Basic Particles"

Generally, the density controlling basic particle concentration shall be as small as possible in order to obtain as high a concentration of the active substance as possible. However, depending on the application, the density controlling basic particles concentration by volume is selected from a group consisting of:

- 1 - 95 %
- 1.5 - 75 %,
- 5 - 50 %,
- 5 - 40 %,
- 5 - 30 %, most preferred.

"Dimensions of Chromatographic Adsorbent Particles"

According to the invention, optimum dimensions of the chromatographic adsorbent particles of the type according to the present invention will largely depend upon the use to which they are to be put, although limitations dictated by the nature of the material and/or by the nature of the active substance and conglomerating agent within the chromatographic adsorbent particles may also play a role.

From the point of view of achieving the greatest rate of interaction of chemical species with a given mass of conglomerate of a particular type, it will generally be advantageous that the total surface area of the chromatographic adsorbent particles is as large as possible, and thus that the size of the chromatographic adsorbent particles is as small as possible.

In preferred aspects of the chromatographic adsorbent particles according to the invention, the size of substantially all of said chromatographic adsorbent particles is within a range selected from the group consisting of:

- 1-10000 μm ,
- 1- 5000 μm ,
- 1- 4000 μm ,
- 1- 3000 μm ,
- 1- 2000 μm ,
- 1- 1000 μm ,
- 50- 500 μm .

The actual size range preferred is dependent on the actual application and the desired control of the dispersion properties, e.g. sedimentation and floatation, of the chromatographic adsorbent particles both properties being dependent on the density and the size range of the chromatographic adsorbent particles. Thus, for very fast separation flow rates chromatographic adsorbent particles of relatively low or high densities and relatively large sizes are preferred. However, large chromatographic adsorbent particles may be limited in diffusion in certain applications, e.g. when proteins have to diffuse in and out of the chromatographic adsorbent particles and interact with active substances within the chromatographic adsorbent particles.

For purification and binding of proteins and other high molecular weight substances which may diffuse slowly in the chromatographic adsorbent particles, e.g. in the conglomerating agent, the preferred size of the chromatographic adsorbent particles is within a range selected from the group consisting of:

1-2000 μm ,
 10-1000 μm ,
 50- 750 μm ,
 100- 500 μm , most preferred.

5 For chromatographic adsorbent particles within the context of the present invention to be of use, for example, in chromatographic separation processes, the time-scale of the process of diffusion of the liquid phases through the chromatographic adsorbent particles, where relevant, should preferably be short in order to ensure sufficiently rapid equilibration between extra- and intraparticle phases; this time-scale will often be of the order of seconds.

10

(b) Density Controlling Basic Particles and Materials

In selecting density controlling basic particles for use as low or high density particles according to the invention, the material of the particles depends on the purpose. Generally, the material is to be sought
 15 among amorphous silica, quartz, or glass.

"Siliceous Glassy or Ceramic Materials"

As mentioned earlier, the prior art discloses a number of examples of hollow particles of siliceous
 20 glassy or ceramic material which may be used as low density hollow particles of chromatographic adsorbent particles according to the invention, these previously disclosed particles being obtained relatively cheaply and straightforwardly by deliberate synthesis or as a fly-ash by-product of certain combustion processes.

Accordingly, in a further preferred aspect of the invention, the material of the basic particles employed
 25 in chromatographic adsorbent particles of both low and high density particles according to the invention is a glass, preferably a synthetic glass comprising silicon dioxide and/or a silicate.

In yet another preferred aspect of the invention, such material is a silicon dioxide-containing material derived from fly-ash, in which case the material may be amorphous (e.g. glassy) or crystalline, or to some extent both amorphous and crystalline.

30

"Structure of Density Controlling Basic Particles"

Further, the material of the density controlling basic particles within the context of the present invention may be chemically and/or physically inhomogeneous. For example, it may have a layered structure
 35 involving one or more layers of similar or different materials, e.g. various types of siliceous materials.

(c) Active substances

Concerning the active substances covalently bound to the chromatographic adsorbent particles accord-
 40 ing to the invention, this may, for example, be any type of material which is useful for a given application.

According to the invention the material of an active substance comprises a member selected from the group consisting of organic and inorganic compounds or ions, non-metallic elements, and organic polymers of biological and synthetic origin.

It is preferred that the active substance comprises a member selected from the group defined in claim
 45 11.

"Introduction of Active Substance into the Chromatographic Adsorbent Particles"

Generally, the active substance may be introduced into the chromatographic adsorbent particles in a
 50 number of ways depending on the nature of the active substance, the conglomerating agent, and the chromatographic adsorbent particles, e.g. their pore size. Thus, both low and high molecular weight ligands may be incorporated during conglomeration either by chemical cross-linking or by copolymerisation. Further, both low and high molecular weight ligands may be chemically coupled to a conglomerating agent before or after conglomeration, or they may be coupled to precursor monomers or polymers introduced
 55 together with the conglomerating agent during the conglomeration provided the desired functions of the active substance is kept intact or may be reestablished before use. However, if the means of conglomeration damages or destroys the functioning of the active substance, the fragile active substance may be introduced after conglomeration provided the conglomerate has been designed with suitable pore sizes

to allow access to its interior.

"Pore Sizes and Their Formation"

5 The optimum size or size-range of the through-going pores will, of course, vary very considerably, depending on the use to which the permeable chromatographic adsorbent particles is to be put. Such pore sizes are difficult to characterize quantitatively; however, in terms of the size of the molecules which are to be capable of passing through the pores, a realistic upper exclusion limit for macromolecules, notably biological macromolecules, such as proteins, will often be a molecular weight of the order of magnitude of 10⁸. The practical lower limit for pore size will generally be set by physico-chemical considerations, e.g. the detailed chemical structure of the outer part and the manner in which the outer part material dissolves or reacts during the pore-formation process.

Pore sizes may typically be formed by methods known *per se*, e.g. by simply controlling the concentration of the conglomerating agent. Thus, for agarose derivatives a larger concentration will provide a smaller pore size. However, other methods may be applied depending on the conglomerating agent and e.g. the incorporated polymers and copolymers.

"Activation or Derivatization"

20 In cases where the conglomerating agent may not have the properties to function as an active substance, the conglomerating agent, or agents, or polymers introduced in the conglomerate, may be derivatized to function as one or more active substances by procedures of activation or derivatisation well known *per se*. Thus, materials comprising hydroxyl, amino, amide, carboxyl or thiol groups may be activated or derivatized using various activating chemicals, e.g. chemicals such as cyanogen bromide, divinyl sulfone, epichlorohydrine, bisepoxyranes, dibromopropanol, glutaric dialdehyde, carbodiimides, anhydrides, hydrazines, periodates, benzoquinones, triazines, tosylates, tresylates, and diazonium ions.

(d) Conglomerating Agents

30 In selecting the conglomerating agent for use as a means of keeping the density controlling basic particles together and as a means for binding the active substance, the conglomerating material is to be sought among certain types of natural or synthetic organic polymers, such as defined in claim 1.

"Organic Polymers"

35 In one aspect of the invention the material of the conglomerating agent comprises a member selected from the group consisting of organic monomers and polymers of biological and synthetic origin, preferably as defined in claim 8.

40 "The Active Substance as Conglomerating Agent"

In one aspect of the invention the conglomerating agent can function as the active substance. In a further aspect, the conglomerating agent may further in a mixture comprise a member selected from the group consisting of:

45 natural and synthetic polynucleotides and nucleic acids, including DNA, RNA, poly-A, poly-G, poly-U, poly-C and poly-T; and

natural and synthetic peptides and polypeptides and other amino acid based polymers, including gelatins, albumins, hemoglobulins, immunoglobulins including poly- and mono clonal antibodies, antigens, protein A, protein G, lectins, glycoproteins such as ovomucoids, and biotin binding proteins e.g. avidin and streptavidin; and

50 other materials used as active substance provided they can conglomerate the density controlling basic particles.

"Activation or Derivatization of Conglomerating Agents"

55 In cases where the conglomerating agent may not have the properties to function as an active substance, the conglomerating agent may be derivatized to function as one or more active substances by procedures of activation or derivatisation well known *per se*. Thus, materials comprising hydroxyl, amino,

amide, carboxyl or thiol groups may be activated or derivatized using various activating chemicals, e.g. chemicals such as cyanogen bromide, divinyl sulfone, epichlorohydrine, bisepoxyranes, dibromopropanol, glutaric dialdehyde, carbodiimides, anhydrides, hydrazines, periodates, benzoquinones, triazines, tosylates, tresylates, and diazonium ions.

(e) Illustration of Chromatographic Adsorbent Particles

Fig. 1A shows a 40X amplified photograph of aspherical chromatographic adsorbent particles 10 having 1-2 mm of diameter and being prepared by distributing unicellar glass microspheres 11 in conglomerating agarose 12 according to Example 1(a).

Fig. 1B shows a 40X amplified photograph of selected spherical chromatographic adsorbent particles also prepared according to Example 1(a).

(f) Fluid Bed Reactors

"C Reactor"

Fig.2 illustrates a cross section of a preferred embodiment of a fluid bed reactor 20 composed of a outer cylinder 21, a top lid 22 with inlet 221 and connection for a stirrer 222, and a bottom lid 23 with outlet 231. Further, an inner cylinder 24 having holes and mounted on a support 25 attached to support blocks 251 and 252 allowing passage of the fluid. Stirring is performed at a suitable rate of rotation within the inner cylinder 24 to assure a sharp lower boundary 26 of the fluid bed chromatographic adsorbent particles. Without stirring, the bed of light conglomerates float against the top lid 22 and have a lower boundary 27.

"T reactor"

Fig. 3 illustrates a cross section of another preferred embodiment of a fluid bed reactor 30 similar to the reactor illustrated in Fig.2 except that the inner cylinder 24 is replaced by an inverse funnel 34 having an upper outlet 341 and supported by a support cylinder 35 that is open upwards. Chromatographic adsorbent particles arriving below the funnel in the turbulens free volume will rise up through the upper outlet 341 while fluid flow down through the outlet 231. The stirrer is placed right below the surface 36 and stirring is performed at a suitable rate of rotation to provide a sharp lower boundary 37 of the chromatographic adsorbent particles. Without stirring, the bed of light chromatographic adsorbent particles have a lower boundary 38.

"Controlled Fluid Distribution in Fluid Bed Reactors"

Fig. 4A and 4B show perspective sketches of a preferred embodiment of a down flow fluid bed reactor 40.

A dc-motor 41 controlled by a variable speed control 42 provides revolutions of a stirrer 43, which in a turbulent zone A agitates the fluid bed particles to generate a turbulent flow of the fluid flowing down-ward.

A sharp interface (generally of few particle diameters) is reached at the non-turbulent zone B in which the particles are stationary and an even and smooth distribution of the fluid is obtained. In order to adapt the conditions of agitation the length of the fluid bed column can be changed by means of interchangeable chromatographic tubes 45.

(A) "Down-Flow Fluid Bed Reactor"

Fig. 5 shows a longitudinal section of a segment of a down-flow fluid bed reactor 50 comprising a vertical cylinder 54 and a fluid bed A,B,C of particles 51,52,53 suspended in a down-flow fluidizing fluid 56 let in through an inlet at the top of the reactor vessel, the particles 51,52,53 having a specific gravity less than that of the fluid. A gas head 57 is above the surface along the line VIA-VIA.

The upper part of the fluid bed is agitated by a plate formed mechanical stirrer 55 dividing the bed into a turbulent zone A, a non-turbulent zone B, and an exit zone C.

In the turbulent zone A, the agitated fluid bed particles 51 move vigorously which generates a turbulent flow of the fluid. The turbulence decreases down the turbulent zone A. A sharp interface VIC-VIC is reached at the non-turbulent zone B in which the particles 52 are in a stationary fluidized state. Across the interface VIC-VIC, the fluid flow is distributed evenly, and a smooth fluid flow is obtained in the non-turbulent zone B.

In the exit zone C, the pooled reacted and/or unreacted fluid 57 leave the fluid bed at an interface VID-VID, where particles 53 can become separated from the fluid bed by the fluid flow.

Figs. 6A-6C show cross sections of the mixing zone A along the lines VIB-VIB, VIC-VIC, and VID-VID, respectively, of the Fig. 5. Thus, Fig. 6A shows a cross section of essentially randomly moving particles 51, and Fig. 6B & 6C show cross sections of essentially stationary fluidized particles 52 and 53. Fig. 6D shows a cross section, along the line VIE-VIE, essentially without particles.

5. EXAMPLES

All solutions employed in the following examples are aqueous solutions unless otherwise indicated.

EXAMPLE 1

Preparation of conglomerates for chromatographic adsorbent particles according to the invention based on 3M's unicellarglass microspheres "Glass Bubbles", B28/750, C15/250, and E22/400, [soda-lime-borosilicate] having a mean density of 0.28 g/cm³, 0.15 g/cm³, and 0.22 g/cm³, respectively.

(a) Low Density Agarose Hollow Glass Spheres Conglomerated Particles

300 ml soya bean oil was heated together with 3 ml sorbitane sesquiolate to 60°C. 5 ml 6% agarose (HSA, Litex) in water was heated and 0.5 g hollow glass spheres (3 M, B28/750) having a mean density of 0.28 g/cm³ were added under stirring. Following mixing of the agarose and glass microspheres the suspension was added to the soya bean oil under heavily stirring. The emulsion formed was stirred at about 60°C in five minutes and cooled to 20°C. The solidified agarose particles containing basic particles of hollow glass spheres were washed on a sintered glass filter with sufficient ether until all soya bean oil was removed. The conglomerate was then washed with water. The conglomerate had a low density and was floating on water.

(b) Low Density Agarose Hollow Glass Sphere Conglomerated Block Polymer Particles

300 ml 4% agarose was prepared by heating 12 g agarose (HSA, Litex) in 300 ml water. 9 g hollow glass spheres (C15/250, 3M) was added and the mixture was stirred until a homogeneous suspension was obtained. The suspension was cooled to 60°C under steadily stirring and the fluid suspension poured on to an efficiently cooled surface. The agarose glass sphere suspension was gelated over a short period. The gel block had a homogeneous distributed content of hollow glass spheres. After cooling the gel block was blended and the granulate was sorted according to size and flow ability by means of "reverse sedimentation".

(c) Low Density Gelatin Hollow Glass Sphere Conglomerated Particles (not according to the invention) - Illustration of Density Control

Five samples of 100 ml 5% gelatin (35°C) in 0.15 M sodium chloride were added hollow glass spheres (E22/400, 3M) in increasing amounts:

- A: 0 g
- B: 2 g
- C: 5 g
- D: 20 g
- E: 27 g

After adjustment of pH to 5.5 all samples were added 2.0 ml glutaric dialdehyde (25% solution, cat.no.: 820603, Merck) under thorough stirring. After 24 hours of incubation at room temperature the polymerized matrices were disintegrated in a blender. The resulting particles were separated from fines by reverse sedimentation (for A by sedimentation as these particles were not floating). The particles were then collected on a glass filter and drained for excess water by vacuum suction on the glass filter. The wet but drained particles were then weighed and the particle volume determined by adding a known amount of liquid followed by determination of the total volume. The following particle densities were obtained:

Measured Density:		Calculated Density:
A:	1.0 g/ml	1.00 g/ml
B:	0.9 g/ml	0.93 g/ml
C:	0.8 g/ml	0.85 g/ml
D:	0.6 g/ml	0.63 g/ml
E:	0.5 g/ml	0.57 g/ml

(d) Low Density Gelatin Hollow Glass Sphere Conglomerated Particles, and Immobilization of Horse-Radish Peroxidase (not according to the invention) – Illustration of Size Range Control

1 g of horse-radish peroxidase (grade II, Kem-En-Tec, Denmark) was dissolved in a solution of 100 ml 10% gelatin (cat.no.: G-2500, Sigma) and 0.5 M sodium chloride (35 °C). 10 g hollow glass spheres (B28/750, 3M) were added under stirring. After adjustment of pH to 5.5, 2 ml glutaric dialdehyde (25% solution, cat.no.: 820603, Merck) was added with thorough stirring. The resulting gel was incubated at room temperature for 2 hours and then disintegrated in a blender. The floating particles were separated from fines and non-floating particles by inverse sedimentation. The yield of wet, packed particles was approx. 120 ml. The size range was determined to be from about 200 to about 500 µm in diameter.

(e) Low Density Agar–Gelatin Glass Sphere Conglomerated Particles I

2 g agar (Bacto-agar, Difco), and 3 g gelatin (cat.no.: G-2500, Sigma) was dissolved in 100 ml 0.15 M sodium chloride by brief heating to the boiling point. After cooling to about 56 °C 10 g hollow glass beads (B28/750, 3M) were added. pH was adjusted to 4.0 with 5 M acetic acid followed by the addition of 2 ml glutaric dialdehyde (25% solution, Cat. No. 820603, Merck) with thorough stirring. The resulting polymer block was cooled to room temperature and incubated for 24 hours followed by disintegration in a blender.

Floating particles were separated from fines and non-floating particles by inverse sedimentation followed by collection of the floating particles on a glass filter. The yield of floating conglomerate particles was 95 ml packed wet particles.

(f) Low Density Agar–Gelatin Glass Sphere Conglomerated Particles II

2 g agar (Bacto-agar, Gibco) and 3 g gelatin (cat.no.: G-2500, Sigma) was dissolved in 100 ml 0.15 M sodium chloride by brief heating to the boiling point. After cooling to about 56 °C 10 g hollow glass beads (B28/750, 3M) were added. The suspension was then cooled by pouring it onto an ice-cold glassplate. The resulting gel block was incubated for 24 hours at 4 °C followed by disintegration by blending in ice-water. The conglomerate floating gel-particles were separated from non-floating particles by inverse sedimentation and then collected on a glass filter. The yield was 105 ml of packed, wet particles.

The particles were then suspended in 200 ml 0.1 M potassium phosphate buffer pH 6.5 and crosslinked for two hours by addition of 10 ml glutaric dialdehyde (25% solution, 820603, Merck).

(g) Low Density Chitosan Glass Sphere Conglomerated Particles

A 4% solution of chitosan (Cat. No.: 22741, Fluka) was prepared by heating 12 g chitosan in 300 ml 10% v/v acetic acid. The viscous solution was cooled to about 40 °C followed by addition of 20 g hollow glass beads (B28/750, 3M). 3 ml glutaric dialdehyde was added (25% solution, 820603, Merck) with thorough stirring. The resulting polymer block was incubated for 24 hours at room temperature followed by disintegration in a blender.

The conglomerate floating gel-particles were separated from non-floating particles by inverse sedimentation in 0.1 M sodium chloride and then collected on a glass filter. The yield was 400 ml of packed, wet particles with a diameter from about 200 µm to about 800 µm.

EXAMPLE 2*Chemical Derivatisation of Low Density Agarose Glass Sphere Conglomerated Particles Prepared According to Example 1 (a)(b)*

5 10 g (dried, wet weight) agarose conglomerate spheres containing hollow glass spheres from Example 1 were suspended in 100 ml 0.5 M potassiumphosphate/sodiumhydroxide pH 11.4. 10 ml divinyl sulfon og 50 mg sodiumborohydrid were added under stirring. The suspension was stirred at room temperature for three hours and the spheres were washed with water on a glass filter. The spheres were then activated 10 chemically (i.e. a method out of many possibilities) and were ready for coupling of other substances. As an example mercaptoethanol was coupled for salt-dependent chromatopgraphy: The spheres were reacted with 5% mercaptoethanol in water that had been titrated to pH 9.5 with 1 M sodiumhydroxide for 3 hours at room temperature.

The spheres were then washed thoroughly with distilled water and were ready to be used as 15 chromatographic adsorbent particles in purification of proteins using salt-dependent chromatopgraphy.

EXAMPLE 3*Purification of Human Immunoglobulin from Untreated Blood Using Chromatographic Adsorbent 20 Particles Prepared According to Example 2*

100 g (dried, wet weight) divinylsulfon and mercaptoethanol treated agarose conglomerate spheres equilibrated with and suspended in 50 ml 0.75 M ammoniumsulphate were placed in a cylindrical glass column with an inner diameter of 5 cm and length of 10 cm. The glass column was sealed at the top and 25 bottom using unscrewing plastic caps. The bottom lit had an outlet with a tube piping in the middle while the top lit had a corresponding inlet and a mechanical stirrer. The mechanical stirrer provides stirring through a air tight collar for stirring the conglomerate spheres contained in the column. The stirring propeller was designed to avoid fluid flow that carries the agarose conglomerate spheres down to the outlet in the bottom column. 2 l unfiltrated and not centrifugated human blood (i.e. outdated blood from a blood 30 bank) having been added ammoniumsulphate to a final concentration of 0.75 M is lead through the column from the top with a flow of 10 ml/min under stirring with the abovementioned stirrer (i.e. to avoid the formation of channels through the fluid bed). 2000 ml of 0,75 M ammoniumsulphate was added at the same flow rate for washing non-bound proteins and particulates. Finally, the bound proteins were eluted from the conglomerate spheres by leading 500 ml of 0.1 M sodium chloride through the column.

35 About 5 g human immunoglobulin was eluted in the sodium chloride fraction. Qualitative analysis showed a high purity of immunoglobulin having a very small contamination of albumin (< 1%).

A corresponding purification of immunoglobulins with divinylsulfon and mercaptoethanol treated agarose spheres without hollow glass spheres was not possible in a traditionally packed column because of clogging 40 of the column by the red blood cells and other sticky materials in blood plasma.

EXAMPLE 4*Immunsorption Using Chromatographic Adsorbent Particles Prepared According to Example 2*

45 Agarose conglomerate spheres containing 4% agarose and produced as described in Example 1 were activated with divinylsulfon as described in Example 2.

10 g (drained, wet weight) activated gel was coupled to rabbit immunoglobulin by incubation of the gel over night with 20 ml rabbit immunoglobulin solution (10 mg immunoglobulin/ml in 0.1 M sodiumhydrogen- carbonate/sodium-hydroxide buffer, pH 8.6 and 5% w/v polyethylenglycol MW 20,000). Excess active 50 groups were blocked by incubation of the gel with 0.5 M ethanolamine/HCl, pH 9.0 for three hours. The gel was coupled with more than 80% of the added rabbit immunoglobulin.

The floating conglomerate spheres having rabbit immunoglobulin attached could then be applied in an apparatus corresponding to the one in Example 3 for adsorption of anti-bodies against rabbit im- munoglobulin from untreated serum of previously pure rabbit immunoglobulin immunized goats. The 55 separated antibody was of a purity and activity corresponding to that obtained with conventionally packed columns using filtered and centrifuged antiserum.

EXAMPLE 5***Immobilised Enzyme. Immobilization of Glucose Oxidase on Chromatographic Adsorbent Particles Prepared According to Example 2***

10 g divinylsulfon activated agarose conglomerate spheres from Example 2 were mixed with 20 ml of a solution of glucose oxidase from *Aspergillus niger* (10 mg/ml in 1 M potassiumhydrogenphosphate/sodiumhydroxide buffer, pH 10.5). The mixture was left for three hours and the uncoupled glucose oxidase was washed out of the spheres by 1 M sodium chloride.

The enzyme coupled conglomerate spheres showed glucose oxydase activity with glucose as a substrate. The development of hydrogen peroxide was detected as a brown colouring of the gel and solution by coupling the reaction with peroxidase (horse-radish peroxidase) oxidation of orthophenylen diamine.

EXAMPLE 6***Chromatographic Adsorbent Particles Comprising Immobilized N-acetylglucosamine for the Separation of Wheat Germ Agglutinin.***

Conglomerate spheres containing 4% agarose and produced as described in Example 1 (b) were activated with divinyl sulfon as described in Example 2. 10 g (dried, wet weight) of the activated gel was coupled to N-acetylglucosamine by incubating the gel over night with 20 ml 0.5 M potassium-phosphate/sodiumhydroxide buffer pH 11.5 containing 50 mg N-acetylglucosamine per ml. Following incubation the excess of active vinyl groups were blocked by 5% mercaptoethanol titrated to pH 9.5 by sodium hydroxide. The gel was washed thoroughly with 1 M sodium chloride. The binding capacity for wheat germ agglutinin was larger than 10 mg lectin per ml gel.

EXAMPLE 7***High Density Gelatin Solid Glass Sphere Conglomerated Particles (not according to the invention) - Illustration of Density Control***

Four samples of 100 ml 5% gelatin in 0.15 M sodium chloride (35°C) were added solid glass spheres (0.075-.15 mm, Fryma, Switzerland) with a density of 2.5 g/ml in increasing amounts:

- A: 10 g
B: 50 g
C: 100 g
D: 200 g

After adjustment of pH to 5.5 all samples were added 2.0 ml glutaric dialdehyde (25% solution, Cat. No.: 820603, Merck) under thorough stirring. After 24 hours of incubation at room temperature the polymerized matrices were disintegrated in a blender. The resulting particles were separated from fines by sedimentation. The particles were then collected on a glass filter and drained for excess water by vacuum suction on the glass filter. The wet but drained particles were then weighed and the particle volume determined by adding a known amount of liquid followed by determination of the total volume. The following particle densities were obtained:

Measured Density:		Calculated Density:
A:	1.1 g/ml	1.06 g/ml
B:	1.3 g/ml	1.25 g/ml
C:	1.5 g/ml	1.43 g/ml
D:	1.7 g/ml	1.67 g/ml

Claims

1. Chromatographic adsorbent particles having covalently bound at least one active substance for binding molecules in a liquid chromatographic fluid bed process;

said adsorbent particles being constituted by a porous composite material having pores allowing access to the interior of the composite material of said molecules;

CHARACTERIZED in

(a) that the porous composite material consists of a conglomerate having controlled relative density; said conglomerate consisting of:

(i) at least two density controlling basic particles of amorphous silica, quartz, or glass selected from the group consisting of low density particles having a density providing floatation and high density particles having a density providing sedimentation of the conglomerate in said liquid; and (ii) a matrix formed by consolidating at least one conglomerating agent selected from the group consisting of natural and synthetic polysaccharides and other carbohydrate based polymers with the proviso that the conglomerating agent is not β -1,3-glucan;

said at least two density controlling basic particles being dispersed in said matrix;

(b) that the size range of the adsorbent particles is controlled;

(c) that said density and said size range are selected to provide desired floatation/sedimentation properties of said adsorbent particles in the liquid in said fluid bed process; and

(d) that the at least one active substance is covalently bound to said matrix.

2. Particles according to claim 1, CHARACTERIZED in that said basic particles are impermeable to the fluid.

3. Particles according to any one of claims 1 or 2, CHARACTERIZED in that the low density basic particles are hollow.

4. Particles according to any one of claims 1 or 2, CHARACTERIZED in that the high density basic particles are solid.

5. Particles according to any one of claims 1-4, CHARACTERIZED in that the basic particles constitute from 1 to 95 %, generally from 1.5 to 75 %, particularly from 5 to 50 %, preferably from 5 to 40 %, and most preferably from 5 to 30 %, by volume of the conglomerate.

6. Particles according to any one of claims 1-5, CHARACTERIZED in that the low density basic particles consist of hollow particles, or unicellar glass micro spheres.

7. Particles according to any one of claims 1-5, CHARACTERIZED in that the high density basic particles consist of glass particles, preferably glass micro spheres.

8. Particles according to any one of claims 1-7, CHARACTERIZED in that the natural and synthetic polysaccharides and other carbohydrate based polymers are selected from the group consisting of:

a) agar, alginate, carrageenan, guar gum, gum arabic, gum ghatti, gum tragacanth, karaya gum, locust bean gum, xanthan gum, agaroses, celluloses, pectins, mucins, dextrans, starches, heparins, chitosans, hydroxy starches, hydroxypropyl starches, carboxymethyl starches, hydroxyethyl celluloses, hydroxypropyl celluloses, and carboxymethyl celluloses; and
b) mixtures of these.

9. Particles according to any one of claims 1-8, CHARACTERIZED in that the natural and synthetic polysaccharides and other carbohydrate based polymers are agarose.

10. Particles according to any one of claims 1-9, CHARACTERIZED in that the active substance comprises a material or mixtures of materials selected from the group consisting of:
organic and inorganic compounds or ions, non-metallic elements, and organic polymers of biological and synthetic origin.

11. Particles according to any one of claims 1-10, CHARACTERIZED in that the active substance comprises a member selected from the group consisting of:

ligands known *per se* in the field of chromatography e.g. charged species i.a. for ion exchange chromatography, proteins, dyes, enzyme inhibitors, specific ligands for specific proteins e.g. biotin for purification of avidin and other biotin binding proteins, carbohydrates for purification of lectins or glycosidases, protein A, chelates e.g. iminodiacetic acid, amino acids e.g. arginine, lysine, and

histidine, sulfated polymers including e.g. heparins, benzhydroxamic acid, hydrophobic ligands e.g. hydrocarbon groups such as phenyl, thiophilic ligands i.e. divinyl sulfone activated substances coupled with mercaptoethanol, 4-hydroxy-pyridine, 3-hydroxy-pyridine, or 2-hydroxy-pyridine;

5 natural and synthetic polynucleotides and nucleic acids, including DNA, RNA, poly-A, poly-G, poly-U, poly-C and poly-T;

natural and synthetic polysaccharides and other carbohydrate based polymers, including agar, alginate, carrageenan, guar gum, gum arabic, gum ghatti, gum tragacanth, karaya gum, locust bean gum, xanthan gum, agaroses, celluloses, pectins, mucins, dextrans, starches and heparins;

10 natural and synthetic peptides and polypeptides and other amino acid based polymers, including gelatins, albumins, hemoglobulins, immunoglobulins including poly- and mono clonal antibodies, antigens, protein G, lectins, glycoproteins such as ovomucoids, biotin binding proteins e.g. avidin and streptavidin, enzymes e.g. proteases, and protease inhibitors; and mixtures of these.

15 12. Particles according to any one of claims 1-11, CHARACTERIZED in that the active substance is covalently bound to the adsorbent particles by means of activation or derivatisation agents activating or derivatizing the conglomerating agent, or the conglomerate particles.

20 13. Particles according to claim 12, CHARACTERIZED in that the activation or derivatisation agents are selected from a group consisting of:

cyanogen bromide, divinyl sulfone, epichlorohydrine, bisepoxyranes, dibromopropanol, glutaric dialdehyde, carbodiimides, anhydrides, hydrazines, periodates, benzoquinones, triazines, tosylates, tresylates, and diazonium ions.

25 14. Particles according to any one of claims 1-13, CHARACTERIZED in that the relative density is from 0.1 to 15, particularly from 0.1 to 5, and preferably from 0.2 to 2.

15. Particles according to any one of claims 1-13, CHARACTERIZED in that the relative density is from 1 to 15, particularly from 1.1 to 5, and preferably from 1.1 to 2.

30 16. Particles according to any one of claims 1-13, CHARACTERIZED in that the relative density is from 0.1 to 1, generally from 0.2 to 0.95, particularly from 0.3 to 0.8, and preferably from 0.5 to 0.75.

35 17. Particles according to any one of claims 1-16, CHARACTERIZED in that their size is within a range selected from the group consisting of:

1-10000 μm ,
1-5000 μm ,
1-4000 μm ,
1-3000 μm ,
40 1-2000 μm ,
1-1000 μm ,
50-500 μm .

45 18. Particles according to claim 17, particularly for purification and binding proteins and other high molecular weight substances, CHARACTERIZED in that their size is within a range selected from the groups consisting of:

1-2000 μm ,
10-1000 μm ,
50-750 μm ,
50 100-500 μm , most preferred.

19. A method of preparing chromatographic adsorbent particles as defined in any one of claims 1-18, CHARACTERIZED by

55 a) mixing basic particles selected from the group consisting of low density particles having a density providing floatation, and high density particles having a density providing sedimentation of the conglomerate in the liquid, said basic particles being made of amorphous silica, quartz, or glass with at least one conglomerating agent selected from the group consisting of natural and synthetic polysaccharides and other carbohydrate-based polymers as defined in claim 8.

- and optionally heating said mixture;
 b) emulsifying said mixture in a suitable solvent;
 c) consolidating said conglomerating agent by a suitable means such as gelation by heating/cooling,
 polymerisation of monomer or monomer mixtures, and non-covalent or covalent cross-bonding; and
 d) isolating and washing the consolidated conglomerate particles.
20. A method according to claim 19, CHARACTERIZED in that the isolated and washed conglomerate particles are sorted according to size and flow ability.
21. A method according to claim 19 or 20, CHARACTERIZED in that the conglomerating agent or the conglomerate particles are activated or derivatized.
22. A method of preparing chromatographic adsorbent particles as defined in any one of claims 1-18, CHARACTERIZED by
 a) mixing basic particles selected from the group consisting of low density particles having a density providing floatation, and high density particles having a density providing sedimentation of the conglomerate in the liquid, said particles being made of amorphous silica, quartz, or glass with at least one conglomerating agent selected from the group consisting of natural and synthetic polysaccharides and other carbohydrate-based polymers as defined in claim 8 and optionally heating said mixture;
 b) consolidating said conglomerating agent by a suitable means such as gelation by heating/cooling, polymerisation of monomer or monomer mixtures, and non-covalent or covalent cross-bonding;
 c) disintegrating the block of conglomerate;
 d) segregating and washing the conglomerate particles.
23. A method according to claim 22, CHARACTERIZED in that the segregated and washed conglomerate particles are sorted according to size and flow ability.
24. A method according to claim 22 or 23, CHARACTERIZED in that the conglomerating agent or the conglomerate particles are activated or derivatized.
25. The use of chromatographic adsorbent particles according to any one of claims 1-18, including β -1,3-glucan as a conglomerating agent, as a solid phase matrix in a fluid bed reactor.
26. The use according to claim 25 in a procedure selected from a group consisting of: chromatographic procedures applying non-packed columns including liquid chromatography, ion-exchange chromatography, and biospecific affinity chromatography such as immunosorption and protein A chromatography, and group specific affinity chromatography such as hydrophobic, thiophilic, dye, lectin, and metal chelate chromatography.
27. The use according to claim 25 or 26, CHARACTERIZED in that the fluid bed reactor is a liquid down-flow fluid bed reactor comprising a vertical reactor vessel with an inlet, an outlet, a fluid bed of said chromatographic adsorbent particles, and agitation means; said agitation means being located near or in the fluid bed proximal to the liquid inlet.
28. The use according to claim 27 for distributing the liquid in the fluid bed, CHARACTERIZED in
 a) that the chromatographic adsorbent particles and liquid proximal to the liquid inlet are agitated to divide the fluid bed into
 i) a turbulent zone having vigorously moving particles, and
 ii) a non-turbulent zone;
 said non-turbulent zone adjoining said turbulent zone; and
 b) that the extent of said turbulent zone is determined by a degree of agitation selected within a range from
 i) a degree of agitation providing turbulence only in the uppermost part of the fluid bed,
 ii) to a degree of agitation providing turbulence of the particles throughout the fluid bed.
29. The use according to claim 25 or 26, CHARACTERIZED in that the fluid bed reactor is a liquid up-flow fluid bed reactor comprising a vertical reactor vessel with an inlet, an outlet, a fluid bed of said

chromatographic adsorbent particles, and agitation means; said agitation means being located near or in the fluid bed proximal to the liquid inlet.

30. The use according to claim 29 for distributing the liquid in the fluid bed, CHARACTERIZED in

- 5 a) that the chromatographic adsorbent particles and liquid proximal to the liquid inlet are agitated to divide the fluid bed into
 - i) a turbulent zone having vigorously moving particles, and
 - ii) a non-turbulent zone;
- 10 b) that the extent of said turbulent zone is determined by a degree of agitation selected within a range from
 - i) a degree of agitation providing turbulence only in the lower-most part of the fluid bed,
 - ii) to a degree of agitation providing turbulence of the particles throughout the fluid bed.

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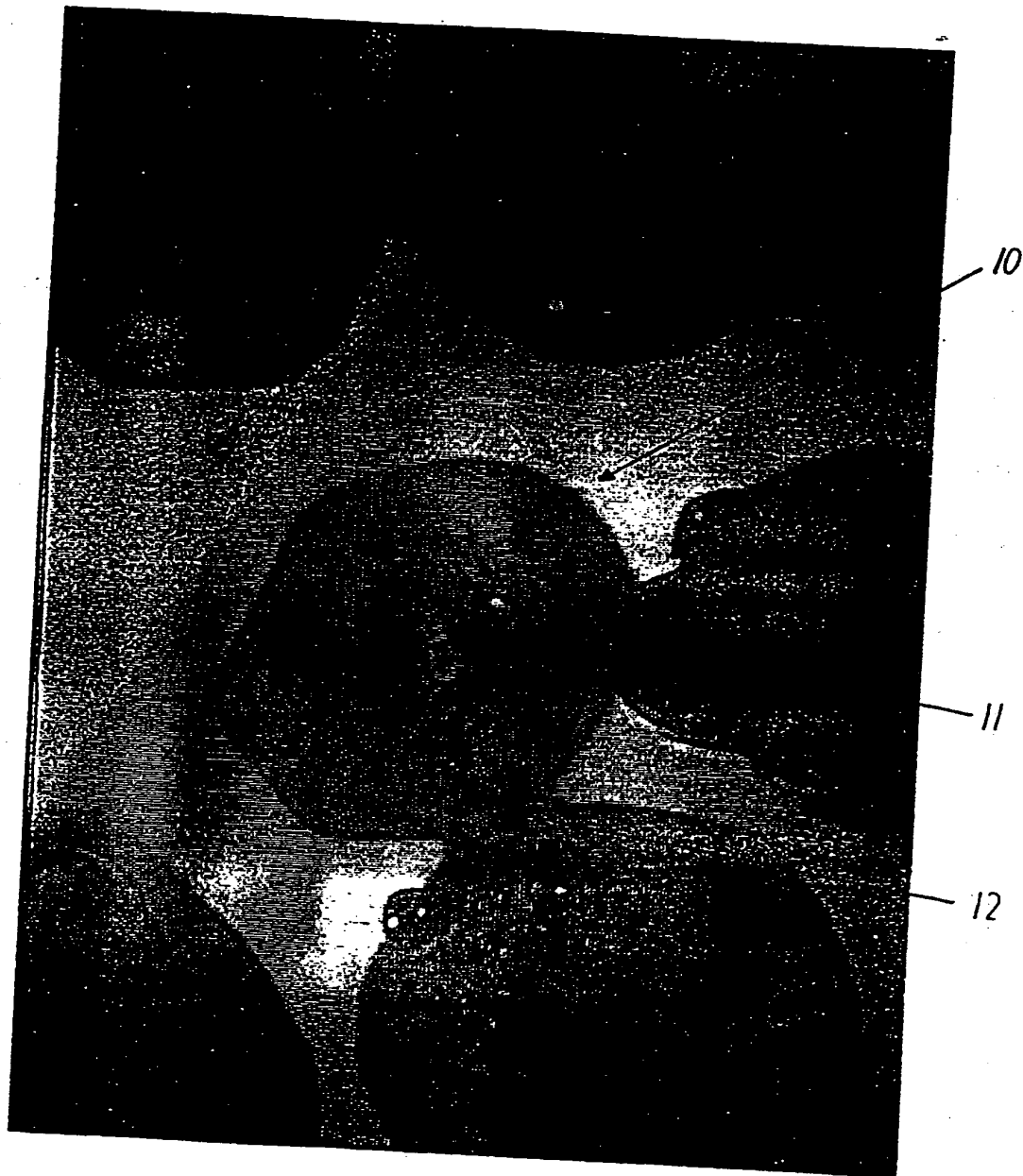


FIG. 1A

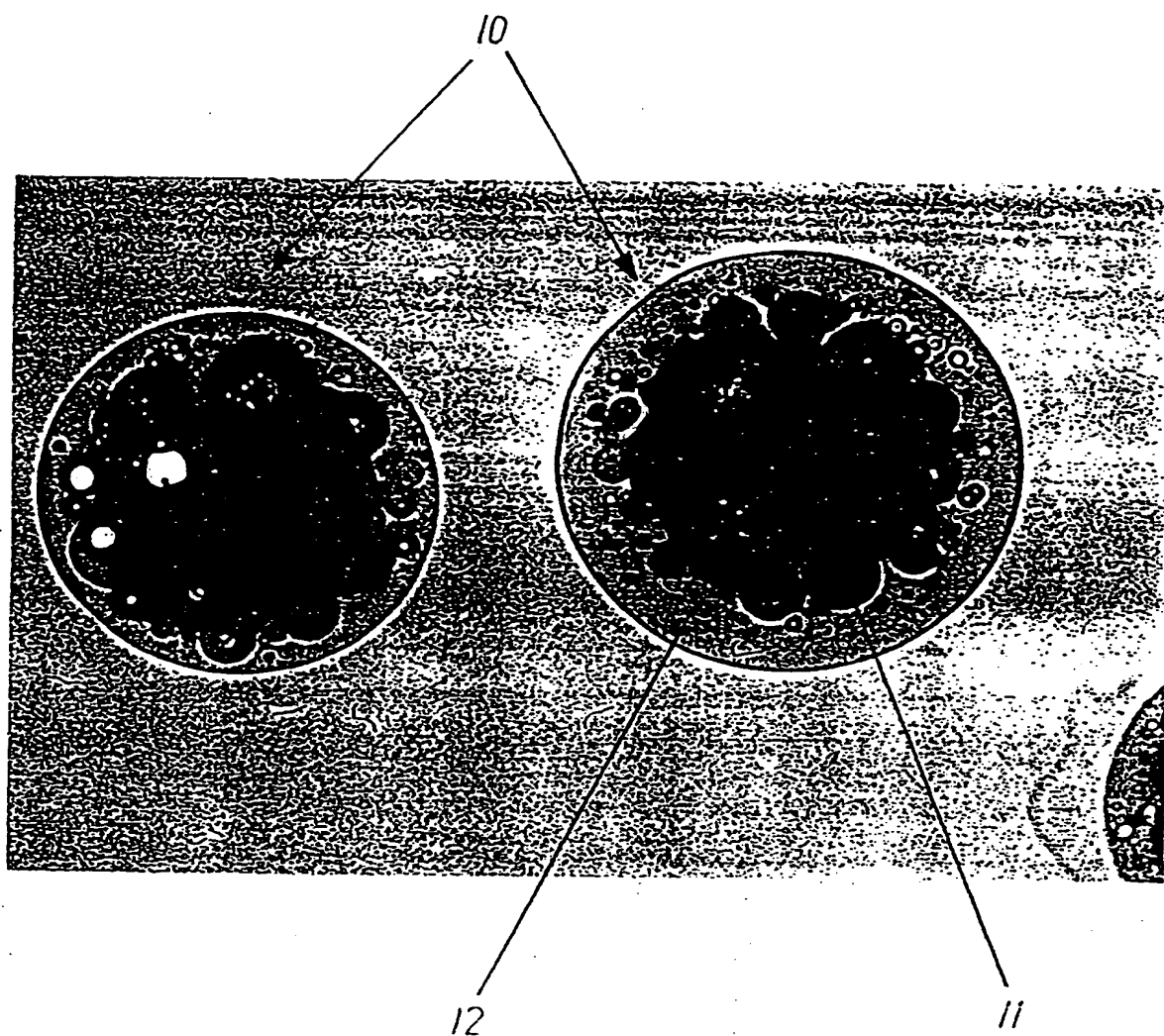


FIG. 1B

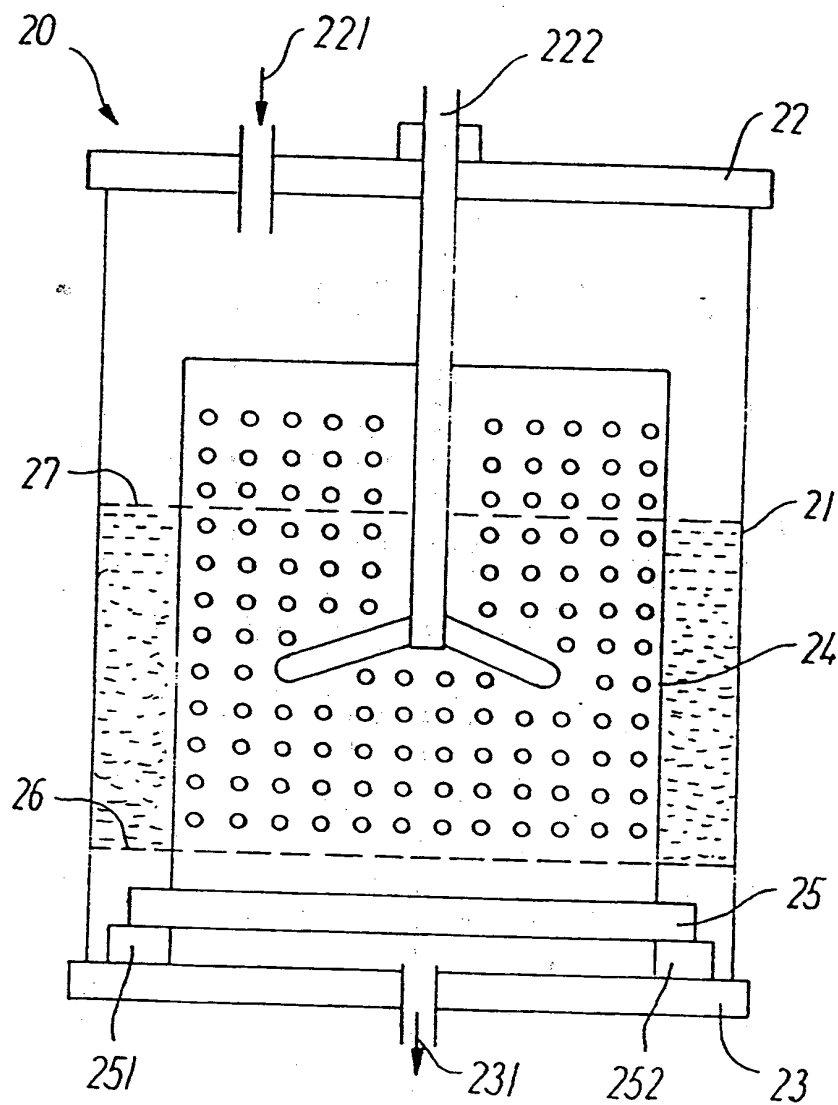


FIG 2

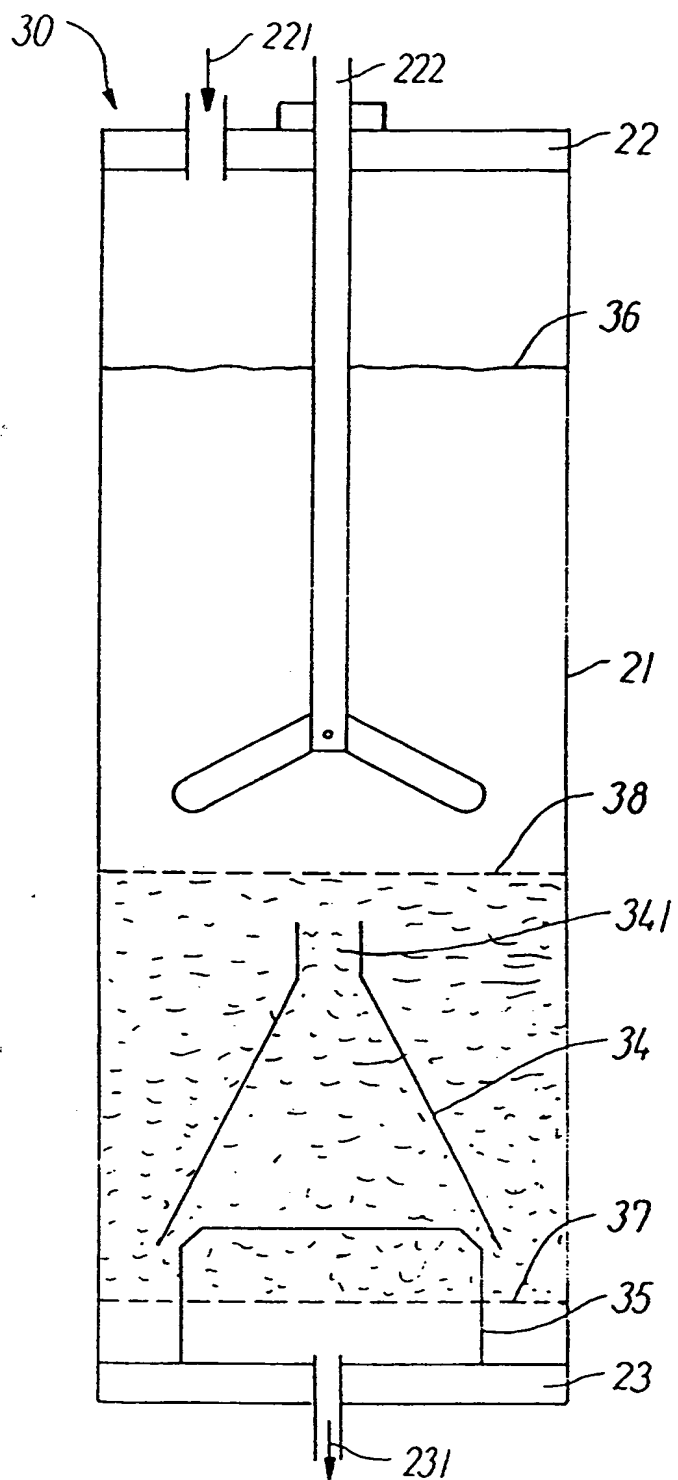


FIG. 3

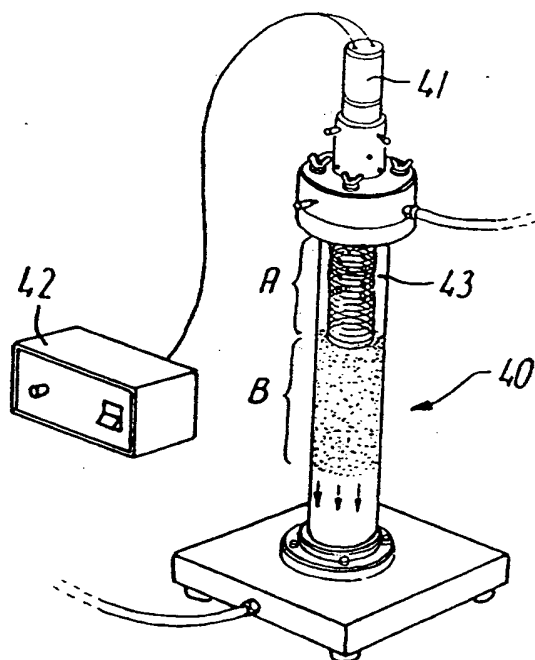


FIG. 4A

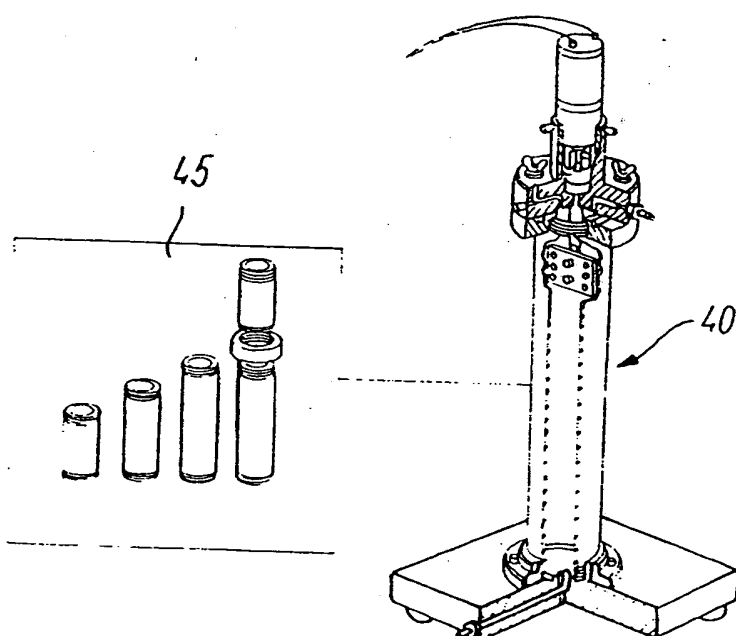


FIG. 4B

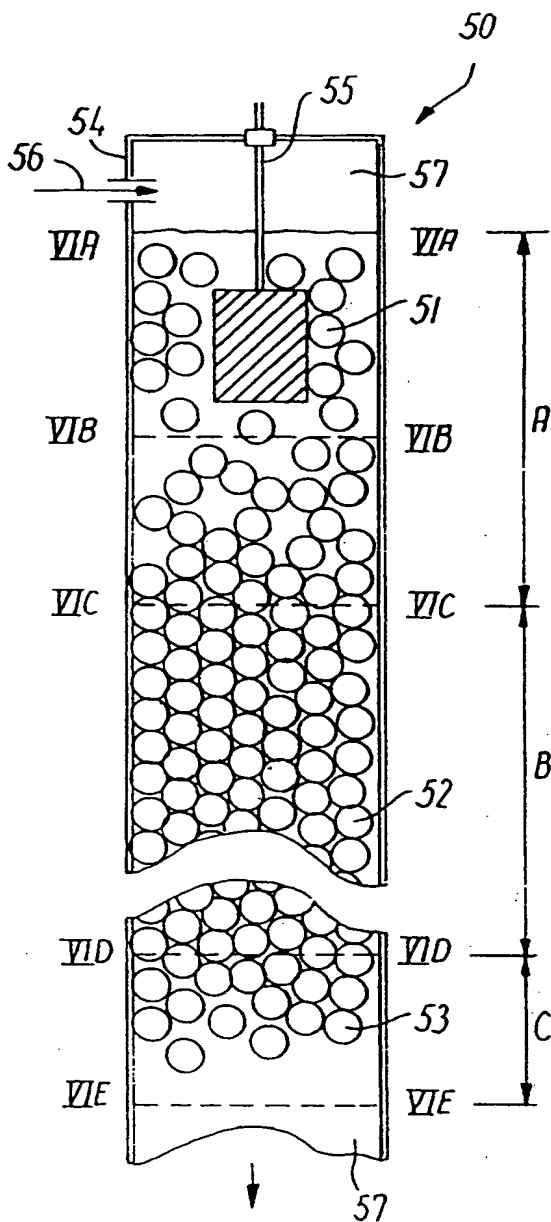


FIG. 5

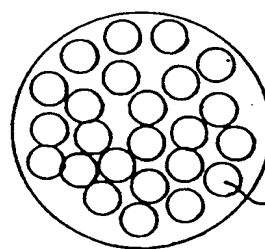


FIG. 6A

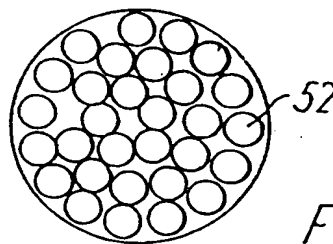


FIG. 6B

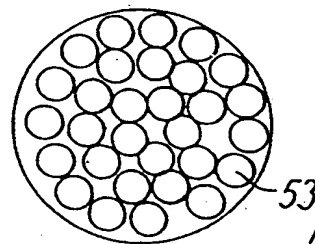


FIG. 6C

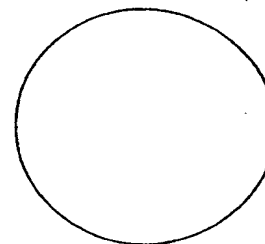


FIG. 6D

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MDA/BSH



Application No. 00 912 411.6 - 2113	Ref. 22816 EP 01	Date 17.03.2004
Applicant UPFRONT CHROMATOGRAPHY AS		

Invitation pursuant to Article 96(2) and Rule 51(2) EPC

Further examination of the above application has revealed that, for the reasons given in the enclosed copy of the result of consultation by telephone on 11.03.2004, it does not meet the requirements of the European Patent Convention.

You are requested to remedy the indicated deficiencies within a

period of 4 months

from notification of this invitation.

The time limit is calculated in accordance with the provisions of Rule 78(2), 83(2) and (4) EPC.

Failure to reply to this invitation in due time will result in the European application being deemed to be withdrawn (Article 96(3) EPC).



DE BIASIO A

For the Examining Division

Enclosure(s):

Copy of result of consultation (Form 2036)
EP-0607998A

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Application No. :

00 912 411.6

Consultation by telephone with the applicant / representative

Despatch with a time limit of 4 month(s)

Participants

Representative: DALKIAEL M

Member(s) of the
Examining Division: DE BIASIO A

Result of consultation

see Annex (Form 2906)



11.03.2004

.....
Date

DE BIASIO A

.....
Examiner

Enclosure(s):
EP-0607998A

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Datum
Date 17.03.2004
Date

Blatt
Sheet 1
Feuille

Anmelde-Nr.:
Application No.: 00 912 411.6
Demande n°:

The applicant was told that the examining division was still of the opinion that the subject-matter of claim 1 was not inventive (Art. 56 EPC) over WO-A-97/17132 (D1):

D1, on page 22, ll. 23-25, teaches to use particulate material ("beads") having a diameter $< 80 \mu\text{m}$ in order to obtain a high mass transfer. D1 also discloses that the density of these particles should be at least 1,2 g/ml. It is also known from D1 that density is an important parameter to consider for particulate material in fluidised and expanded beds (page 4, ll. 12-17).

The examining division further introduced EP-A-0607998 into the proceedings, a copy is annexed to the present communication. The examining division pointed out that in claim 15 of EP-A-0607998 relative densities of 1.1 to 5 were disclosed and that it appeared that a density of 2,5 g/ml was a value the skilled person would contemplate when applying the technical teaching of prior art.

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